FINAL TECHNICAL REPORT

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Identification of Inhibitory Components in Dilute Acid Pretreated Lignocellulosic Materials

Richard F. Helm, Judith Jervis and Thilini D. Ranatunga
Fralin Biotechnology Center
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061-0346

INTRODUCTION

The work performed in this time period concentrated on ascertaining what methods, besides overliming, can improve the fermentability of xylose-rich prehydrolyzates with *Zymomonas mobilis* CP4(pZB5). Difficulties at NREL in providing ion-exchanged material concomitant with the departure of the research associate in charge of the project at Virginia Tech (Thilini Ranatunga), seriously hampered our rate of progress. Nonetheless, the experiments completed provide a relatively clear picture of where the toxicity resides and what protocols, besides overliming, generate a fermentable product. These results are discussed in relationship to our future efforts at ascertaining the mechanism of overliming.

Background Information

The Final Technical Report submitted in May 1998[1] summarized the work completed on a series of heat-treated yellow-poplar hydrolyzates that had been further processed by anion and cation exchange, as well as overliming. Also included in the report were a couple of recommendations for future work:

1. Analysis and Testing of a C_{18} Purified Aqueous Fraction. Experiments carried out through May of 1998 indicated that the uronic acids and the organic fractions in the HT or HT/IX hydrolyzates were not significantly toxic to *Zymomonas*. These experiments also indicated that overliming was not effective in reducing any of the toxic effects caused by furans or the organic fraction removed from the hydrolyzate. Therefore, the aqueous fraction recovered after the MTBE extraction should contain some highly toxic materials which are being removed or detoxified during the overliming process. Further analyses needed to be carried out on this fraction.

2. *Assessing the Toxicity of Sulfates toward Zymomonas.* Fermentation studies carried out using synthetic hydrolyzates indicated that the sulfate levels present in the hydrolyzate could be toxic to *Zymomonas*. Thus, fermentation experiments using a synthetic hydrolyzates containing glucose, xylose, and different levels of sulfate ions were suggested.

These two research areas were investigated as part of Progress Reports E[2] and F.[3] Work performed subsequent to Report F (September 1998—June 1999) was concerned with analysis and processing of an NREL-generated untreated hydrolyzate using a combination of ion-exchange and C_{18} solid phase extraction protocols. This work, as well as the work described in Reports E and F, are the subject of this Final Technical Report.

Assessing the Toxicity of Sulfates toward *Zymomonas mobilis*.

Three synthetic hydrolyzates were prepared using glucose and xylose concentrations similar to that of the Heat-treated/Ion-exchanged material prepared by NREL:

Synthetic Hydrolyzate 1: Glucose and xylose in 0.82% (w/w) H_2SO_4.
Synthetic Hydrolyzate 2: Glucose, xylose, and acetic acid in 0.82% (w/w) H_2SO_4.
Synthetic Hydrolyzate 3: Glucose, xylose, and acetic acid in water.

Bioassays on these synthetic hydrolyzates both directly neutralized and overlimed were compared to a positive control (glucose and xylose in water). The bioassay results are shown in Table 1, and indicated that sulfate ions alone are only mildly toxic to *Zymomonas*, alone or in the presence of acetate. In addition, mixtures of sulfuric and acetic acid had a higher fermentability than the hydrolyzate containing only acetic acid. Based on these results[1,2] it can be concluded that the toxicity caused by sulfate ions in a hydrolyzate is significantly less than that of acetate.

Table 1. Ethanol Yields Obtained After Fermenting with Various Synthetic Hydrolyzates (24 hr fermentation, 50% loading level).

Sample	Ethanol (mg/mL)	Percent Performance[a]
Positive Control[b]	18.28	100
Hydrolyzate 1 (Glc/Xyl/H_2SO_4)		
Direct Neutralized	15.67	86
Overlimed	15.76	86
Hydrolyzate 2 (Glc/Xyl/HOAc/H_2SO_4)		
Direct Neutralized	15.49	85
Overlimed	14.92	82
Hydrolyzate 3 (Glc/Xyl/HOAc)		
Direct Neutralized	13.95	76
Overlimed	12.88	70

Shake flask bioassays with pH adjustment at 0, 3 and 6 hr. [a]Percent performances expressed relative to the positive control. [b] Sugar levels in positive control represent those of the NREL hydrolyzate HT/IX, and all hydrolyzate samples were supplemented with sugars to match this level.

Toxicity Testing of C_{18}-Purified Fractions of the HT/IX Hydrolyzates.

With the solid phase extraction protocol, the non-polar materials are bound to the solid matrix and the aqueous fraction, which contains the sugars, elutes unbound. Fractions obtained from this separation were toxicity tested toward *Zymomonas* using the standard pH-controlled shake flask bioassay. The ethanol yields obtained after a 24 h fermentation is given in Table 2. The performance values indicate that the aqueous fraction does indeed contain components that are inhibitory to *Zymomonas* xylose fermentation. Interestingly, the direct neutralized and overlimed fractions have approximately the same performances. That overliming of the aqueous fraction is not necessary for optimal performance was a positive result of this experiment.

Table 2. Ethanol Yields (24 hr fermentation) Obtained After Fermenting with the Aqueous Fraction (HT/IX hydrolyzate) from the Solid Phase Extraction.

Sample	Ethanol (mg/mL)	Percent Performances[a]
Positive control	20.44	100
Direct Neutralized Aq. Phase (30% level)[b]	15.60	76
Overlimed Aq. Phase (30% level)[b]	15.75	77

[a]Percent performances are expressed relative to the positive control; [b]All samples were supplemented with sugars to match those of the positive control, the sugar composition of the positive control match those of the NREL treated hydrolyzate HT/IX.

We also investigated the toxicity of the non-polar fraction of the solid phase extraction, adding these materials directly to synthetic hydrolyzates (both directly neutralized and overlimed, 30% loading level). The fraction was found to be non-inhibitory in all formulations, with all preparations, with percent performances greater than 90% (relative to a positive control).

Toxicity of NREL Hydrolyzate P980302SD and Comparison with the Fractions Isolated by C18 Solid Phase Extraction.

To assess the efficacy of solid phase extraction on removing toxicity from raw hydrolyzate (*ie.*, not heat treated or ion-exchanged), the NREL untreated hydrolyzate P980302SD was submitted to a C18 solid phase extraction, and bioassays were performed on the aqueous and organic fractions. The results indicated that both the direct neutralized and overlimed aqueous fractions were highly toxic toward *Zymomonas* at a 60% level, with no significant amounts of ethanol produced. On the other hand, synthetic hydrolyzates spiked with the non-polar organics proved to be relatively non-toxic. Thus most of the toxic components are in the aqueous phase, but it is somewhat difficult to separate the effect of acetic acid from other toxic compounds present.

Ion Exchange, Solid Phase Extraction and Toxicity Testing of NREL Hydrolyzate P980302SD #7

Research completed up to this point had indicated that anion exchange chromatography, without overliming, may be a viable detoxification route for the prehydrolyzate. We wished to investigate the process of anion exchange further. Since there were difficulties at NREL in providing us with anion-exchanged hydrolyzate, we received another batch of untreated hydrolyzate in mid-November 1998 (P980302SD #7) from which a project was generated which had five major components:

1. Compositional analysis of the crude material.
2. A portion of the hydrolyzate was neutralized and freeze dried. This material was then resuspended and passed through a C18 column, collecting both the aqueous fraction. This fraction was submitted to compositional analysis and the shake flask bioassay.
3. The aqueous fraction from Step 2 (above) was passed through a series of two ion-exchange columns, first a strong cation exchanger (H^+ form; Dowex 50WX8, 40 mesh), and then a strong anion exchange column(OH^- form, Dowex SBR). The resulting aqueous solution was submitted to compositional analysis and the shake flask bioassay.

4. A portion of the crude hydrolyzate was passed through an anion exchange column (hydroxide form), concentrated (rotoevaporation), and neutralized to pH 6. The resulting aqueous solution was submitted to compositional analysis and the shake flask bioassay.

5. Material from step 4 (above) was passed through the C18 column as described in step 2 (above). The resulting aqueous solution was submitted to compositional analysis and the shake flask bioassay.

The compositional analysis of the samples listed above, along with the overlimed crude hydrolyzate and the C18 eluate samples are shown in Table 3. There are several items worth mentioning here. First is the xylose losses associated with all of the detoxification processes. This is not a desireable event. As far as the losses due to overliming, we assume that two things may be occurring simultaneously. First, base can degrade carbohydrates to furans as well as low molecular weight organic acids.[4] We have always assumed that this is a relatively minor process, and we still believe it is, but nonetheless, it is possible. One way to ascertain if this is occurring is to check for xylose rearrangement products. The process of base degradation of carbohydrates proceeds through equilibrium formation of a 1,2-enediol. Rearrangement back to a pentose or to a ketose can afford D-xylose, D-lyxose or D-xylulose. One could perform analyses looking specifically for D-lyxose and/or D-xylulose.

Table 3. Compositional Analysis of the Samples Generated.

Sample	Glucose mg/ml	Xylose mg/mL	HOAc mg/mL	Furfural mg/mL	HMF mg/mL
Hydrolyzate	8.4	36.8	10.7	2.04	0.40
Overlimed hydrolyzate	10.5	7.2	---[a]	---[a]	---[a]
C18	6.4	29.7	8.0	trace	trace
C18 + overlime	6.3	12.0	8.0	n.d.[b]	n.d.
C18 + mixed bed ion exchange	0.9	2.6	n.d.	n.d.	n.d.
Anion exchange	4.0	14.3	n.d.	n.d.	n.d.
Anion exchange + C18	nd	5.6	n.d.	n.d.	n.d.

[a]Concentration was not determined. [b]None detected.

A second pathway to carbohyrate loss is through entrainment within the overlimed precipitate. Divalent cations form weak non-covalent associations with carbohydrates, and it could be that a significant portion of the carbohydrates reside within the calcium sulfate precipitate. At present, the solid is removed from the treated hydrolyzate by passing the mixture through a sterile filter. This is oftentimes a slow, painstaking process. If fact, we will often have to place the unit under vacuum to improve the filtration rate. It may be worthwhile for us to check the precipitate for the presence of carbohydrate.

There were also significant losses during the anion exchange process. Explanations for this could be the use of a strong anion exchanger instead of a weak exchanger such as the one used by NREL. There could also be losses during solution concentration (rotoevaporation and freeze drying). In the future we will attempt to eliminate these problems by:

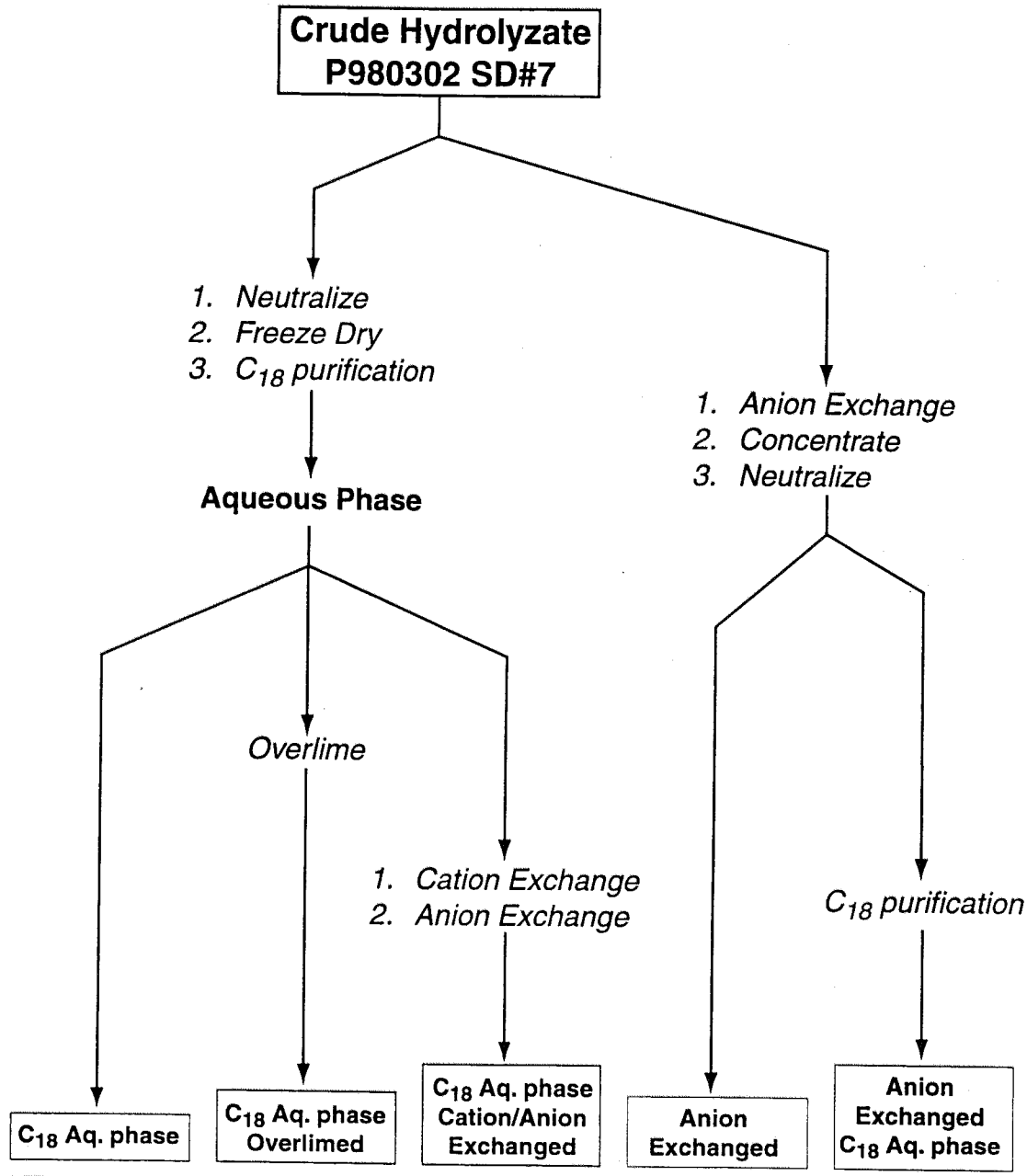
1. Minimizing and/or eliminating rotoevaporation and/or freeze-drying between the different processing stages.
2. Using the same anion exchange matrix as NREL. It may be worthwhile to investigate other anion-exchange resins in the future.
3. Develop a better method for hydrolyzate recovery during overliming and/or eliminate this step.

Bioassays were performed in duplicate on the samples listed in Table 3 (after augmenting solutions to have the appropriate glucose and xylose concentrations), and the results are shown in tabular (Table 4) as well as schematic (Figure 1) form. It seems relatively clear that it is possible to create a highly fermentable product without overliming. These are encouraging results, and need to be corroborated by additional experimentation with samples that have not undergone such significant carbohydrate degradation and/or losses.

Table 4. Fermentability of the Different Hydrolyzate Fractions (30% Loading Level)

Sample	EtOH (mg/mL)	% Performance	Bioassay[a]
Positive Control 1	16.45		
Positive Control 2	16.63		
AVERAGE	**16.54**	100	A
Negative Control 1	1.21		
Negative Control 2	1.31		
AVERAGE	**1.26**	8	A
C18 Eluate 1	8.15		
C18 Eluate 1	8.20		
C18 Eluate 2	8.62		
C18 Eluate 2	8.76		
AVERAGE	**8.43**	51	A
C18 Eluate Overlimed 1	6.56		
C18 Eluate Overlimed 2	6.55		
AVERAGE	**6.56**	40	A
C18 Eluate Ion Exchanged 1	14.23		
C18 Eluate Ion Exchanged 2	14.47		
AVERAGE	**14.35**	87	A
Positive Control 3	17.63		
Positive Control 4	18.99		
AVERAGE	**18.31**	100	B
Negative Control 3	1.63		
Negative Control 3	2.48		
AVERAGE	**1.26**	11	B
Anion Exchange 1	14.80		
Anion Exchange 2	14.54		
AVERAGE	**14.67**	80	B
Anion Exchange/C18 Eluate	16.08		
Anion Exchange/C18 Eluate	16.93		
AVERAGE	**16.51**	90	B

[a]Two sets of bioassays were performed, with a positive and negative control for each set. The values for percent performance are based on the positive control ethanol concentration for each set.



Glucose Recovery*	**77**	**75**	**11**	**48**	**0**
Xylose Recovery*	**82**	**33**	**7**	**39**	**15**
HOAc Remaining*	**75**	**75**	**0**	**0**	**0**
Fermentability*	**51**	**40**	**87**	**81**	**91**

*Value (in percent) relative to the crude hydrolyzate (glucose, xylose, HOAc) or the positive control (fermentability). Negative control (overlimed crude hydrolyzate) fermentability: 10%.

Figure 1. *Schematic representation of the fractions obtained, along with the appropriate mass recovery and fermentability data.*

Summary

It is becoming apparent that there may be alternatives to overliming for the generation of fermentable xylose-rich feedstock. Anion-exchanged hydrolyzates have been observed to be quite fermentable at 30% loading levels without overliming. Furthermore, C_{18} solid phase extraction has been shown to be useful in ascertaining the role of the more non-polar hydrolyzate components. However, we have also seen that the detoxification stategies used have resulted in considerable carbohydrate loss. In the future, we will endeavor to eliminate the recovery issue by concentrating on protocols that do not involve sample concentration at highly acidic or neutral pHs, the use of weaker anion exchange resins, and new protocols to eliminate sugar losses during overliming.

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